Bacterial origin recognition complexes direct assembly of higher-order DnaA oligomeric structures

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Eukaryotic initiator proteins form origin recognition complexes (ORCs) that bind to replication origins during most of the cell cycle and direct assembly of prereplication complexes (pre-RCs) before the onset of S phase. In the eubacterium *Escherichia coli*, there is a temporally similar nucleoprotein complex comprising the initiator protein DnaA bound to three high-affinity recognition sites in the unique origin of replication, *oriC*. At the time of initiation, this high-affinity DnaA–*oriC* complex (the bacterial ORC) accumulates additional DnaA that interacts with lower-affinity sites in *oriC*, forming a pre-RC. In this paper, we investigate the functional role of the bacterial ORC and examine whether it mediates low-affinity DnaA–*oriC* interactions during pre-RC assembly. We report that *E. coli* ORC is essential for DnaA occupation of low-affinity sites. The assistance given by ORC is directed primarily to proximal weak sites and requires oligomerization-proficient DnaA. We propose that in bacteria, DnaA oligomers of limited length and stability emerge from single high-affinity sites and extend toward weak sites to facilitate their loading as a key stage of prokaryotic pre-RC assembly.

DNA replication | *E. coli* | ORC | oriC | pre-RC

Regulating chromosome duplication requires precisely timed formation of nucleoprotein complexes that comprise initiator proteins bound to replication origins and that direct assembly of new replisomes (1–6). Among the best-studied examples of such nucleoprotein complexes are the origin recognition complexes (ORCs) bound to origins in budding yeast (7, 8), and the complexes formed by DnaA binding to the unique origin of chromosomal replication, *oriC*, in *Escherichia coli* (6, 9). Yeast ORC subunits share structural motifs with DnaA as well as archeal Orc1 (9, 10), and all are members of the AAA+ family of ATPases (11). This structural conservation among initiator proteins suggests the intriguing possibility that mechanisms used by all cell types to initiate DNA synthesis could be fundamentally similar (12).

Examination of the binding patterns of initiator proteins to origins during the cell cycle (5, 13, 14) has revealed that in addition to structural similarities, there are temporal similarities in nucleoprotein complex formation at eukaryotic and prokaryotic replication origins. Yeast ORCs bind to replication origins throughout the cell cycle and recruit additional initiator proteins needed to form the prereplicative complexes (pre-RCs) that load helicase and unwind origin DNA before entry into S phase (7, 8, 14, 15). In *E. coli*, a temporally similar nucleoprotein complex is formed by DnaA binding to three high-affinity ($K_d < 200$ nM), 9-bp recognition sites (R1, R2, and R4) within *oriC* (Fig. 1); like yeast ORC, this binding persists throughout the majority of the cell cycle (13, 16, 17), except at the time of initiation, when additional initiator DnaA binds to lower-affinity ($K_d > 200$ nM) sites in *oriC* (13, 18). The additional DnaA causes localized strand separation within an AT-rich, 13-mer repeat region that is adjacent to the left side of the DnaA complex (Fig. 1) (19, 20), followed by recruitment of the helicase loader, DnaC, and the replicative helicase, DnaB, completing the assembly of the bacterial pre-RC (21). Oligomerization-proficient DnaA is required to form the pre-RC (22, 23), and structural analyses have led to the proposal that a DnaA-ATP oligomeric helical filament is responsible for origin unwinding (24).

Based on the structural conservation among initiator proteins, combined with the similarities in cell cycle binding pattern, we recently suggested that the high-affinity DnaA complex could serve as the bacterial version of ORC (13). However, it is not yet known whether the high-affinity complex is functionally similar to yeast ORC in playing a key role in recruiting the additional DnaA to low-affinity sites during pre-RC assembly. In this study, we addressed this question by examining mutant *oriC* lacking high-affinity DnaA recognition sites. We observed that ordered DnaA binding was eliminated in vitro and *oriC* was inactivated in vivo when all high-affinity sites were converted into the weaker R5M site. Restoring individual high-affinity sites to WT allowed limited loading of nearby weak sites (within about 45 bp) as long as DnaA was oligomerization-proficient, suggesting a polymer of limited length emanates from each site. We propose that the *E. coli* ORC regulates ordered and efficient pre-RC assembly by anchoring DnaA oligomers that form on *oriC* DNA between high-affinity sites.

Results

A Mutant *oriC* Lacking High-Affinity Sites Does not Bind DnaA in Vivo. After initiation, *E. coli oriC* rapidly rebinds DnaA to the three high-affinity sites, (13) suggesting that the high-affinity DnaA complex plays an important and early role in rebuilding the pre-RC needed to trigger the next round of chromosome replication. Based on the function of yeast ORC, it seems logical that this role is to facilitate binding of additional DnaA molecules to the lower-affinity sites, and it is logical to predict that a mutant *oriC* lacking high-affinity binding sites should show impaired DnaA-binding capability and reduced function. To test this possibility, we used site-directed mutagenesis to alter *oriC*, converting R1, R2, and R4 to R5M ($oriC^{1,2,4/R5M}$; Fig. 1). $OriC^{1,2,4/R5M}$ was cloned into a drug-resistance plasmid that also contained the pBR322 origin, and *oriC* function was tested by transforming the chimeric plasmid into a *polA* host strain. Because the pBR322 origin requires DNA polymerase I to replicate, colonies arise only when plasmid *oriC* is functional. If the plasmid *oriC* harbors a mutation that makes initiation less efficient, then it will not be able to compete effectively with the chromosomal copy of *oriC* for initiation factors, and the plasmid will not be capable of transforming cells (25, 26). In the competition assay, plasmids harboring $oriC^{1,2,4/R5M}$ were not able to transform *polA* strains, indicating that the function of the mutant *oriC* was impaired sufficiently to prevent it from competing with WT chromosomal *oriC* (transformation efficiencies were less than 0.1% of those obtained by using WT *oriC* plasmids). To determine whether $oriC^{1,2,4/R5M}$ could replicate in the absence of competition, we attempted to use the $oriC^{1,2,4/R5M}$ plasmid to transform a *polA* strain in which chromosomal *oriC* was replaced by the R1 plasmid replication origin (27). Again, no colonies were obtained, indicating that *oriC* plasmid lacking high-affinity sites is not capable of

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The authors declare no conflict of interest.

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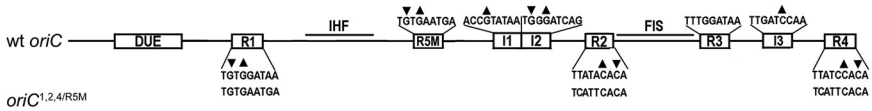

Fig. 1. Map of *oriC*. Locations and sequences of DnaA recognition sites are shown. The mutated sequences in $oriC^{1,2,4/R5M}$ are placed below the WT sequences. Positions of IHF, Fis, and the duplex unwinding region (DUE) are marked. The change in DMS modification pattern caused by DnaA binding is indicated by up (increased modification) or down (decreased modification) arrowheads.

replicating in vivo, even when there is no competition from the chromosomal origin.

Because $oriC^{1,2,4/R5M}$ lacks high-affinity DnaA-binding sites, it is likely that the loss of function seen with this origin is related to a defect in ordered DnaA binding. We used dimethyl sulfate (DMS) footprinting to compare ORC and pre-RC formed in vivo on $oriC^{1,2,4/R5M}$ and WT *oriC*. DMS preferentially methylates guanosines, and DnaA binding reproducibly changes the methylation pattern within recognition sites. Specifically, DnaA binding enhances the methylation of the G in the fourth position of the 9-mer recognition site and suppresses modification of the G in the second position (if present), marked by arrows in Fig. 1. This pattern is easiest to see when comparing increased G4 intensity relative to decreased G2 intensity within a single lane. In vivo, $oriC^{1,2,4/R5M}$ DnaA binding was not observed at any *oriC* site in exponentially growing cells (Fig. 2*A*, lane 3; quantified in *C*). In contrast, on WT *oriC*, DnaA binding was observed at R1, R2, and R4, as we have reported previously (Fig. 2*A*, lane 1; quantified in *B*) (13, 18). To examine the DnaA complex that forms at the time of intiation, *dnaC*(ts) cells harboring plasmids containing either WT *oriC* or $oriC^{1,2,4/R5M}$ were held at a nonpermissive temperature long enough to accumulate sufficient DnaA to align the cells at the stage just before helicase loading. In the aligned cells harboring WT *oriC*, there is enough accumulated DnaA to bind low-affinity R5M and I sites (Fig. 2*A*, lane 2, and *B*). However, in aligned cells harboring $oriC^{1,2,4/R5M}$, the level of DnaA was insufficient to bind the mutant origin at any site (Fig. 2*A*, lane 4, and *C*). This result suggests that R5M and I sites have a lower affinity for DnaA on $oriC^{1,2,4/R5M}$ than they do in the WT *oriC* configuration. Consequently, the data demonstrate that the high-affinity DnaA–*oriC* complex is required for normal loading of the additional DnaA needed to fill lower-affinity sites.

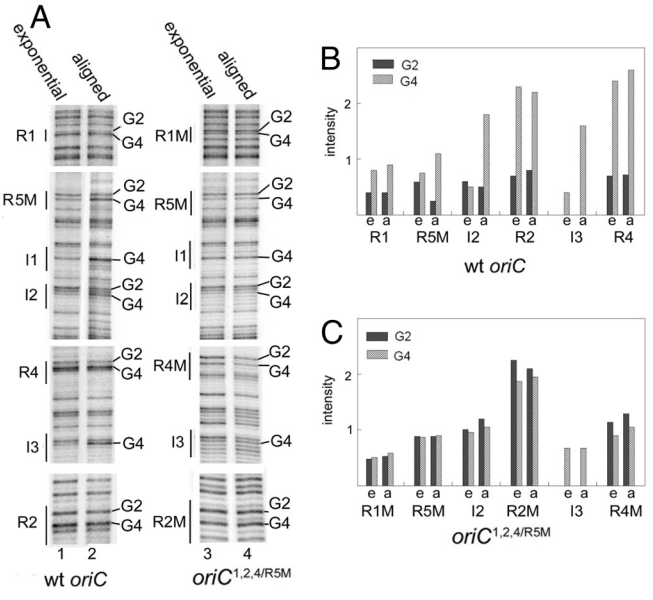

Fig. 2. A mutant *oriC* lacking high-affinity sites does not bind DnaA in vivo. (*A*) *DnaC*(ts) cells harboring either WT *oriC* or $oriC^{1,2,4R5/M}$ were grown exponentially or were held at nonpermissive temperature for 1 h to align them at the stage of initiation just before helicase loading. Cells were treated with DMS, and modification patterns were measured. Positions of DnaA-binding sites and bands representing the Gs at positions 2 and 4 are marked. (*B* and *C*) Relative intensities of DMS-modified guanosines in DnaA-binding sites were quantified from scans of footprinting gels. Quantitation from representative scans is shown.

DnaA Bound to Strong Sites Increases the Affinity of Weak Sites in Vitro. For a more detailed analysis of ordered DnaA binding to $oriC^{1,2,4/R5M}$, we performed in vitro DMS footprinting of $oriC^{1,2,4/R5M}$ and WT *oriC* using a range of DnaA concentrations (Fig. 3*A*). Although in vivo there is apparently enough DnaA present to fill R1, R2, and R4 throughout the cell cycle, in vitro the differing affinities of the DnaA-binding sites result in DnaA filling in the following order: R4 ≥ R1 > R2 > R5M, I2, I3 > R3, as reported previously (Fig. 3 *A*, lanes 1–3, and *B*) (19, 28, 29). In contrast, there was no evidence of ordered DnaA binding to $oriC^{1,2,4/R5M}$ (Fig. 3 *A*, lanes 4–9, and *C*). Instead, all sites became occupied simultaneously at a DnaA level that was 3- to 4-fold higher than that needed to fill low-affinity sites on wt *oriC*.

DnaA Oligomerization Is Required for Binding to Lower-Affinity Sites in *oriC*. The decreased ability of DnaA to bind to $oriC^{1,2,4/R5M}$ suggests that the high-affinity DnaA-binding sites in *oriC* facilitate binding to lower-affinity sites. One likely mechanism for this cooperative binding would be for the stronger sites to accumulate DnaA oligomers and provide DnaA to lower-affinity sites. DnaA has two known oligomerization domains: the N-terminal domain I (23, 30) and the box VII region of domain III (22, 24, 31). The domain III interactions are reported to require DnaA-ATP and to result in the formation of a helical filament (24). It has been shown previously that DnaA-ADP, and DnaA harboring mutations in box VII, are defective in binding to lower-affinity sites in *oriC* (22, 32, 33). To determine whether N-terminal oligomerization is also required for filling of lower-affinity sites, we examined *oriC* binding of a mutant DnaA(W6A), in which a tryptophan essential for oligomerization was replaced with alanine (23). Although WT DnaA and DnaA(W6A) interacted with R1, R2, and R4 similarly, DnaA(W6A) was less efficient at binding the lower-affinity sites R5M, I2, and I3 (Fig. 4 *A*, compare lanes 1–3 with 4 and 5, and *B*), because three to four times more DnaA(W6A) was required to achieve binding similar to that observed by using WT DnaA. Combined with the previously published studies on domain III mutants (22), these data indicate that both domain I and domain III interactions are required for high-affinity R boxes to assist loading of weaker sites.

The results described above are consistent with a model in which high-affinity DnaA boxes act as nucleation sites for oligomer formation. However, when we examined DnaA binding to a DNA fragment containing a single R4 (TTATCCACA) box by EMSA, only one complex was formed, even at high DnaA/DNA ratios (20:1; Fig. 5*A Left*). These results are consistent with there being only a single molecule of DnaA bound to the R box, similar to results reported previously (34). When the DNA fragment contained two DnaA boxes (R4 and R2) spaced 7 bp

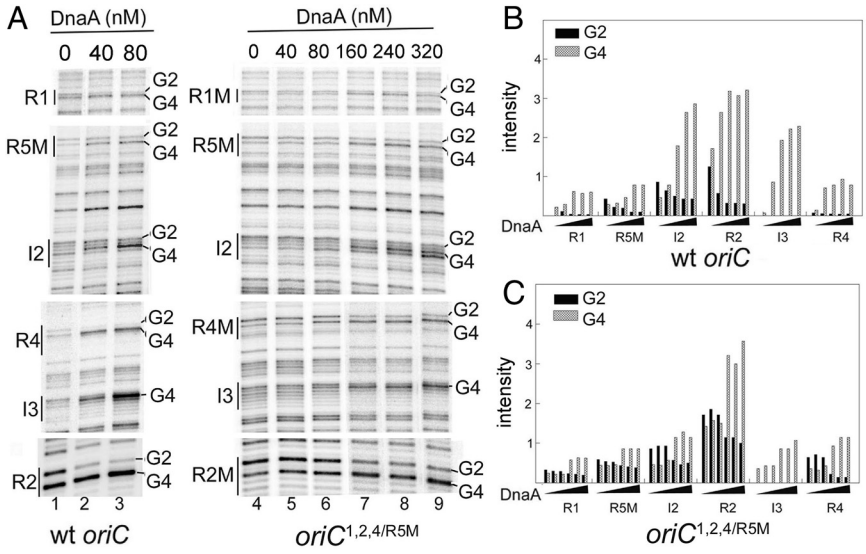

Fig. 3. Strong sites facilitate DnaA binding to low-affinity sites in *oriC*. (*A*) R1, R2, and R4 were replaced by R5M in *oriC* to make $oriC^{1,2,4/R5M}$. DMS modification patterns were measured on WT *oriC* and $oriC^{1,2,4/R5M}$ after incubation with DnaA-ATP. Positions of DnaA-binding sites are marked, and bands representing the Gs at position 2 or 4 are indicated. (*B* and *C*) Relative intensities of DMS-modified guanosines in DnaA-binding sites were quantified from scans of footprinting gels. (*B*) WT *oriC* was incubated with 0, 20, 40, 80, and 100 nM DnaA. (*C*) $oriC^{1,2,4/R5M}$ was incubated with 0, 40, 80, 160, 240, and 320 nM DnaA. Quantitation from representative scans is shown.

apart, two complexes formed, indicating that higher-order complexes are detectable in this assay (Fig. 5*A Right*). These results suggest that DnaA oligomerization might be enhanced by site-specific DNA binding. To demonstrate that formation of DnaA–DnaA multimers is stimulated when DnaA is bound to multiple sites in *oriC*, we combined DnaA-ATP with WT *oriC* or with pACYC184 DNA; the latter should not facilitate DnaA oligomerization because it contains no specific DnaA-binding sites. The samples were then reacted with the cross-linking reagent DSP. DnaA species were revealed by Western blots (Fig. 5*B*), and the extent of multimer formation on each template was compared. We observed very few cross-linked DnaA multimers formed in solution (Fig. 5*B*, lane 1) or on the nonspecific pACYC184 DNA (Fig. 5*B*, lane 2). In contrast, WT *oriC* promoted formation of DnaA multimers, which consisted mostly of dimers and trimers (Fig. 5*B*, lane 3), providing direct evidence that sequence-specific binding of DnaA to adjacent sites promotes DnaA oligomerization. Further examination of the relationship between physical proximity of binding sites and formation of multimers is currently under investigation.

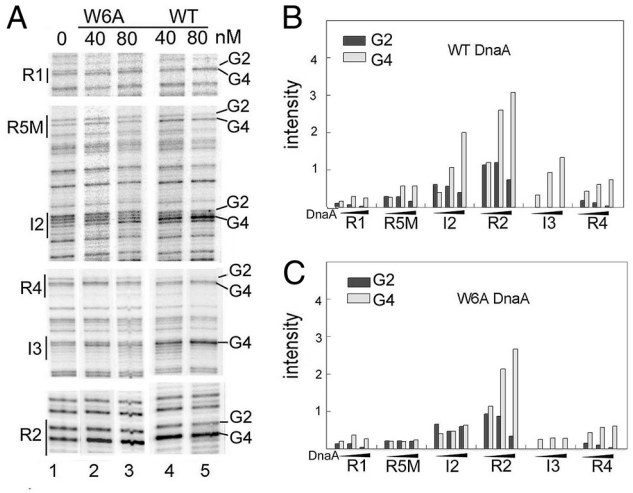

Fig. 4. Oligomerization-proficient DnaA is required for binding to low-affinity sites. (*A*) *oriC* was incubated with the indicated concentrations of WT DnaA or DnaA(W6A) and treated with DMS. Modification patterns are shown. Positions of DnaA-binding sites are marked, and bands representing the Gs at position 2 or 4 are indicated. (*B* and *C*) Relative intensities of DMS-modified guanosines in DnaA-binding sites were quantified from scans of footprinting gels.

Strong DnaA-Binding Sites Donate DnaA to Proximal Weaker Sites. The data presented are consistent with a model in which *E. coli* high-affinity sites act as DnaA accumulation points that provide DnaA to lower-affinity sites, most likely by growth of a DnaA oligomer toward the weak site. If true, a high-affinity site should facilitate loading of the closest lower-affinity site but would have less effect on DnaA occupation of other sites. To examine this, we created origins containing a single high-affinity site in either the R1 ($oriC^{2,4/R5M}$), R2 ($oriC^{1,4/R5M}$), or R4 ($oriC^{1,2/R5M}$) position. None of these mutant origins, harbored on a plasmid also containing a pBR322 origin, were capable of transforming the *polA* strain (transformation efficiency was less than 0.1% of WT *oriC* plasmid), indicating that a single high-affinity site is not capable of accumulating and donating enough DnaA to fill all of the weak sites and initiate DNA replication. To determine whether a single strong site was capable of helping DnaA bind to any of the lower-affinity sites, we performed in vitro DMS footprinting on the mutant origins and evaluated the levels of DnaA required to fill the lower-affinity sites. For WT *oriC*, I2, I3, and R5M bind at essentially the same DnaA level (60 nM; DnaA/*oriC* of 15). However, in $oriC^{2,4/R5M}$, where R1 is the only high-affinity site, R5M, the site closest to R1, filled at a lower DnaA level than the other sites in the origin, suggesting that the intact R1 in this origin was able to donate DnaA most effectively to R5M (Fig. 6*A*). A low level of occupation was observed at the I2 site on $oriC^{2,4/R5M}$ at the higher DnaA levels tested, with detectable site occupation decreasing as sites became more distant from R1 (Fig. 6*D*). (Site occupation can be evaluated by comparing the fold increase in band intensity, measured by the ratio of G4 intensity at the highest DnaA level/intensity with no DnaA.) When DnaA was combined with $oriC^{1,4/R5M}$ (strong site is R2), the occupation pattern shifted and was centered at R2,

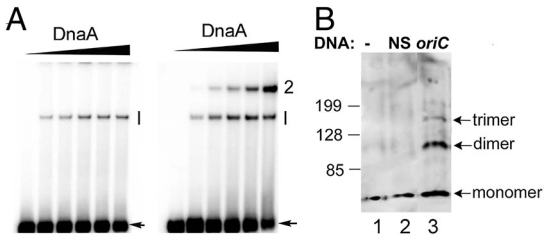

Fig. 5. *oriC* promotes formation of DnaA oligomers. (*A*) A DNA oligomer containing a single R4 box (*Left*) or an R4 box and an R2 box separated by 7 bp (*Right*) were incubated with DnaA-ATP at the indicated molar ratios of DnaA/DNA, and complexes were resolved on polyacrylamide gels. Complexes 1 and 2 are labeled, and arrows point to the unbound probe. (*B*) DnaA-ATP (80 nM) was incubated in the absence of DNA (lane 1) and with nonspecific DNA (NS; pACYC184; lane 2) or *oriC* (lane 3). After incubation, the cross-linking agent DSP was added. The reactions were loaded onto SDS/polyacrylamide gels, and DnaA was revealed by Western blot analyses. Locations of DnaA monomers, dimers, and trimers are marked. Positions of molecular weight markers are indicated on the left.

with a high level of binding observed at I2 and I3 and proportionally decreased binding at more distant sites (Fig. 6 *B* and *D*). Similarly, when DnaA was incubated with $oriC^{1,2/R5M}$ (strong site is R4), binding to R4 and I3 was observed at similar DnaA levels as in WT *oriC*, with little occupation of the other sites at the concentrations used (Fig. 6 *C* and *D*). These results are consistent with each high-affinity site being able to help the loading of proximal low-affinity sites, but this assistance seems to be limited by distance. Combined, the results of these studies support a model of pre-RC assembly in which high-affinity sites first bind single molecules of DnaA and then, as cellular DnaA levels increase, attract additional DnaA to form an oligomer that extends toward the nearest weaker sites.

Discussion

In *E. coli*, assembly of higher-order pre-RC complexes comprising DnaA and *oriC* (4, 6, 35) is exquisitely timed during the cell cycle (36, 37), and so must be orderly, efficient, and reproducible. In yeast and higher eukaryotes, ORCs play critical roles in recruitment and ordered assembly of pre-RC components (8), and it is reasonable to expect that a functional equivalent of ORC also must exist in eubacteria. Here, we present evidence that DnaA persisting throughout the *E. coli* cell cycle at three high-affinity recognition sites in *oriC* constitutes a functional ORC. Eliminating *E. coli* ORC by reducing DnaA affinity at R1, R2, and R4 of *oriC* ORC sites also eliminated all evidence of ordered assembly, even at DnaA levels sufficiently high to occupy all recognition sites on WT *oriC*, suggesting a unique role for ORC in forming a specific higher-order structure. The role for high-affinity DnaA recognition sites in forming the pre-RC is consistent with the finding that loss of strong sites has detrimental effects on *oriC* function (38), and loss of R1 affinity always results in a nonfunctional *oriC* (39). Viability is not lost when origins defective in R2 or R4 are harbored on the chromosome, but initiation timing in these cells is not normal (39), and it is possible that the cell could use alternative but less efficient pathways to build the pre-RC. The requirement for an ORC-like complex bound to bacterial origins for properly timed initiations extends previous observations that many mechanisms regulating replication origin activity are likely to be conserved across all domains of life (10).

Although DnaA-ATP is proposed to form an oligomeric helical filament based on structural features (24), how and when such a filament might form during the cell cycle is not yet known. We observed that an individual strong site was able to promote binding of oligomeric-proficient DnaA to proximal

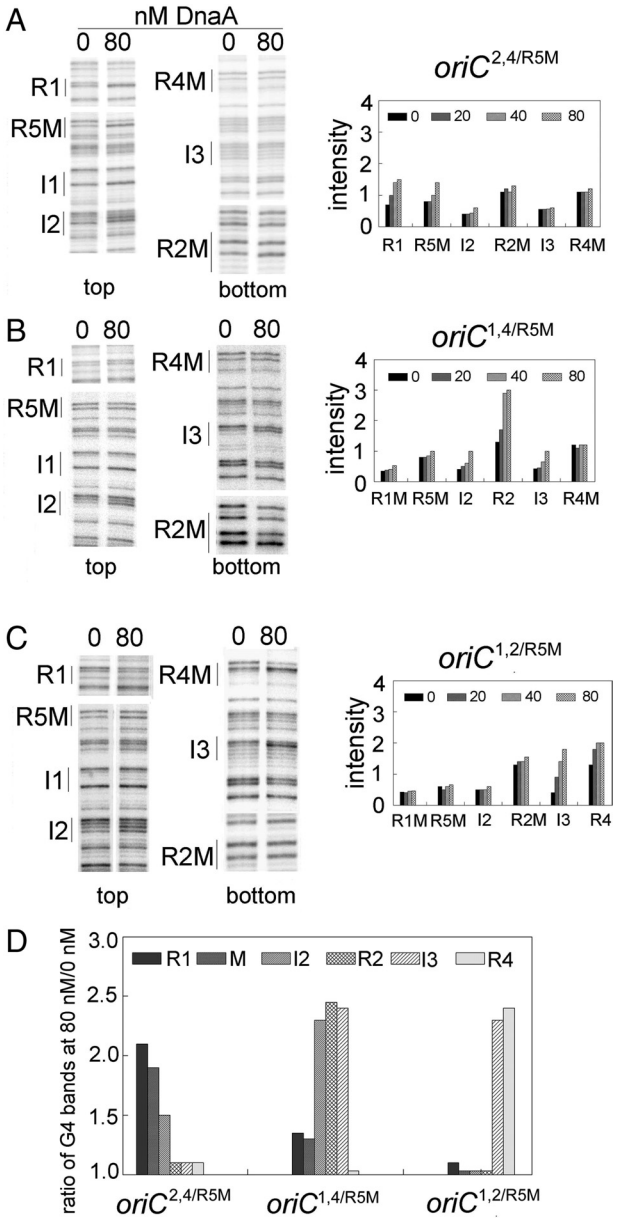

Fig. 6. High-affinity sites facilitate DnaA binding only to proximal low-affinity sites. Mutant *oriC* plasmids containing only one high-affinity site were incubated with the indicated concentrations of DnaA-ATP and treated with DMS. (*A–C*) (*Left*) Scans of footprinting gels with the positions of DnaA-binding sites marked. Mutated sites are indicated by R1M, R2M, or R4M to mark that the respective site was converted to the R5M sequence. (Right) Graphs of the quantitation of relative intensities (arbitrary units) of the G4 in each site. (*A*) $oriC^{2,4/R5M}$ (R1 is the sole high-affinity site). (*B*) $oriC^{1,4/R5M}$ (R2 is the sole high-affinity site). (*C*) $oriC^{1,2/R5M}$ (R4 is the sole high-affinity site. (*D*) The increase in intensity of G4 in each site in the three single-site mutants after incubation of DnaA is shown as the ratio of G4 (80 nM DnaA)/G4 (0 nM DnaA).

pre-RC (low-affinity) sites, suggesting each high-affinity box could anchor extended DnaA oligomers. Based on the three fixed high-affinity site positions in *oriC*, oligomers would need to extend about 95 bases to be anchored by both R1 and R2, and 65 bases for anchoring by both R2 and R4. Placement of low-affinity DnaA-binding sites in the intervening region would allow stabilization of the oligomer between the anchor sites. The use of low-affinity sites to stabilize filaments between relatively distant sites provides an opportunity to reg-

ulate pre-RC assembly by using factors such as Fis, IHF, and SeqA, which act by altering the effective distance between recognition sites by DNA bending, or by blocking access to sites.

There are several features within inter-ORC sequences that support this model. One example is the placement of I sites that preferentially interact with DnaA-ATP between R1–R2 and R2–R4. The strategic placement of I sites would ensure that oligomer assembly was coupled to newly synthesized DnaA-ATP in each half of *oriC*. Another example is the placement of GATC sites into the intervening DNA sequences. Hemimethylated GATC is the recognition site for SeqA protein, previously shown to sequester *oriC* immediately after replication, ensuring initiation only once per cell cycle (40). SeqA is reported to assemble into a filament similar to that proposed for DnaA (41–43), and both filaments are unlikely to coexist at *oriC*. The strategic placement of GATC in the intervening sequences allows high-affinity sites to rebind DnaA to reset the origin, but it prohibits the reassembly of pre-RC by specifically blocking DnaA interactions with I2, I3, and R5M (13). A third example is the placement of recognition sites for DNA bending proteins within the intervening DNA. DNA bending of *oriC* is coordinated during the cell cycle, with rapid switching from a Fis–ORC nucleoprotein complex that represses pre-RC assembly to an IHF-bound complex that redistributes DnaA to pre-RC recognition sites in a DnaA-ATP-dependent fashion (16, 19, 28). IHF places a severe bend into *oriC* (44), moving R1 and R5M into closer proximity and potentially reducing the length of a DnaA-ATP oligomer spanning these sites. DNA bending by Fis prohibits binding of IHF (28), but the Fis-induced bend may also prevent a filament extending from R4 to be able to contact R2.

Although reversing orientation of high-affinity sites or disrupting the helical phasing between strong sites was previously shown to perturb *oriC* function (38, 45), the mechanism for this disruption, particularly in the context of the transition of ORC to pre-RC, has not been evaluated. Both orientation and phasing may play a role in formation of oligomers emerging from strong sites if contact with specific low-affinity recognition sites is required. Increasing the number of strong sites beyond the WT number might also be expected to have profound effects on *oriC* function, and our preliminary studies on mutant versions of *oriC* with four high-affinity sites suggest that these origins overinitiate. Further examination of mutant *oriCs* with altered recognition site placement is likely to provide additional clues to the role of site proximity in the assembly of pre-RC.

We and others have observed that pre-RC assembly and initiation require DnaA oligomerization activities in both N-terminal (domain I) and ATP-binding domains (domain III) (22–24, 30–32). Although it remains to be determined why two oligomerization activities in DnaA are required, all DnaA filaments may simply require both oligomerization domains to assemble a stable higher-order structure, no matter where they exist on *oriC*. Alternatively, the position of high- and low-affinity recognition sites may determine the requirement for a particular oligomerization activity. For example, the distance between two sites that are widely spaced may require N-terminal domain activity, but closely spaced sites may require domain III activities. If this is true, then pre-RC assembly could be divided into different substages based on the type of DnaA–DnaA interactions that were used, with each substage being the target of specific regulatory mechanisms. Such fine-tuning of pre-RC assembly would help ensure that bacterial chromosome replication initiates at a precise time and only once per cell cycle.

Methods

Strains and Plasmids. pOC170 was used as the template for all mutant *oriC* constructions. It carries replication origins from both pBR322 and *oriC* (46). pZL411, which carries an inducible His-10-tagged *dnaA* gene (47), was used to make the W6A DnaA mutation. Supercoiled plasmids were isolated by using the QIAPrep Spin plasmid preparation kit (Qiagen). Site-directed mutagenesis was performed as described previously (33), with all mutations being verified by sequence analysis. For evaluation of in vivo replication of *oriC* plasmids, pOC170 or mutant plasmids were transformed into either P3478 *polA1*, *thyA36 deoC2* IN(rrnD-rrnE)1, λ-, or its isogenic parent, W3110. Evaluation of in vivo DnaA binding was done by using PC2 *dnaC*(ts) harboring either pOC170 or p*oriC*$^{1,2,4/M}$.

Chemicals, Proteins, and Enzymes. Reagent-grade chemicals were purchased from Amresco, Fisher Scientific, or Sigma. Media components were from Difco. All enzymes were from Sigma, New England Biolabs, or Bioline. Amino-terminal His-10-tagged DnaA(wt) and DnaA(W6A) were purified as described by Li and Crooke (47).

DNA Modification and Primer Extension. In vivo DMS modification was done as described previously (19) by using cells grown in minimal media supplemented with glucose and Casamino acids. DMS modification of DNA (0.75 g) in vitro was performed as described previously (33). DnaA was preincubated in reaction buffer with 5 mM ATP for 5 min before addition to reactions at the concentrations indicated in the Figures. All experiments were repeated at least three times. DMS-treated samples were extended with ^{32}P-labeled primer as described previously (33). Two primers were used—a left primer hybridizing at bases 272–290 to analyze top-strand modifications of plasmid template, and a right primer hybridizing at bases 124–142 to analyze bottom-strand modifications. The complete nucleotide sequence of *oriC* is shown in Ryan et al. (18). Extension products were resolved on 6% polyacrylamide sequencing gels, and dried gels were scanned on a Bio-Rad Molecular Imager FX PhosphorImager. Images were analyzed by using Bio-Rad QUANTITY ONE software. Ratios of intensities of bands in binding sites to internal standard bands were calculated to yield relative intensity of modified guanines. Deviations in band intensities among experiments were $<$10%.

Cross-linking Assays. Reactions were set up as for DMS modification, except that no BSA was added to the reaction. After incubation of DNA and DnaA for 5 min, the samples were incubated with dithiobis (succinimidyl propionate) (DSP; 5 μM) for 15 min at 37 °C. Reactions were stopped by the addition of lysine (50 mM). Loading buffer lacking reducing agent was then added to the samples, and the samples were resolved on 8% PAGE/SDS gels. The proteins were electrophoretically transferred to nitrocellulose, and the DnaA on the blots was revealed by using an anti-DnaA antibody (gift from Elliott Crooke), detected by chemiluminescence (Bio-Rad ImunoStar kit).

EMSAs. Binding reactions were the same as for DMS, except that they also contained 1 mM EDTA and 0.2% Triton X-100. Poly(dI-dC) was used as a noncompetitive inhibitor. End-labeled template (5′-CAGTCATTGGC-CGCGTCTCG CTTCCTGACAGAG TTATCCACAGTAG ATCGCA-3′ or 5′-CAGT-CATTGGTTATACACAGCTAGAGTTATCCACAGTAGATCGCA-3′; 0.2–5 nM) was incubated with varying concentrations of DnaA-ATP (DnaA/*oriC* of 0.5 to 20) for 10 min at 20 °C and applied to 6% native polyacrylamide gels running in Tris-borate-EDTA buffer. Dried gels were scanned on a Bio-Rad Molecular Imager FX PhosphorImager.

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